TITLE: METHOD OF IDENTIFYING CHANGES IN BIOPOLYMERS

FIELD OF THE INVENTION

The present invention relates to a method of identifying changes in biopolymers, especially in chromosomal DNA, using two or more different sets of labelled detector molecules, as well as to a diagnostic kit for detecting these changes.

BACKGROUND OF THE INVENTION

The representation of human chromosomes has been carried out so far with banding techniques which permit a specific recognition of the chromosomes using light and dark bands (*e.g.* G-banding, O-banding, R-banding). These banding techniques are based on methods developed by Caspersson *et al.*, (Exp. Cell Res. 80, 1970, 315-319), Sumner *et al.* (Nature 232, 1971, 31), Seabright *et al.* (Lancet 2, 1971, 971-972) and Dutrillaux *et al.* (C R Acad. Sci., Paris, 272, 1971, 3636-3640). However, the identity of individual chromosomal bands can not be defined in every instance with these methods since all bands of all chromosomes appear only either light or dark. This turns out to be a significant disadvantage since chromosomes can be very different morphologically from cell to cell and from tissue to tissue and can possibly comprise translocations (*e.g.*, in the case of tumors) the recognition of which can be of particular significance for the person to be examined. This applies, *e.g.*, to the decision whether or not to have children in the case of a parent having balanced translocations ("crossing-overs" or "exchanges of parts of chromosomes"), to the recognition of the cause of abnormalities in children with and without mental retardation, and to the diagnosis of leukemias and other tumors

which frequently exhibit specific chromosomal changes with diagnostic and therapeutic significance.

Fluorescence in-situ hybridization (FISH) was described for the first time for routine use in practicable form by Pinkel *et al.* (Proc. Natl. Acad. Sci. USA 83, 1986, 2934-2938) as a suggestion for solving this problem. Today, all human chromosomes of a metaphase can be represented in different colors with this method by using chromosome-specific DNA libraries (chromosome painting, 24-color FISH, Schröck et al., Science 273, 1996, 496-497; Speicher et al., Nature Genet. 12, 1996, 368-376). Through the use of vectors, *e.g.*, cosmids, pacs or YACs, which can contain different amount of human DNA, specific chromosomal regions can be re-checked with respect to their integrity via multicolor techniques by means of FISH. Even parts of genes and repetitive DNA elements can be identified in this way regarding their chromosomal localization and their presence or absence. However, a multicolor representation of chromosomal sections at the band level has not been possible so far.

Thus, the problem underlying the current invention is to provide a novel and improved method for the identification of, in particular, changes in chromosomal DNA which method enables a multicolor representation at the band level.

This problem is solved by the embodiments of the present invention characterized in the claims.

DETAILED DESCRIPTION OF THE INVENTION

In particular, a method of identifying changes in biopolymers as target molecules using two or more different sets of labelled detector molecules is provided in which at least two sets

are specific for a certain region in the target molecules and the labels of the particular detector molecules of these sets specific for a certain region in the target molecules are different, the method comprising the steps of

(a) carrying out bonding reactions between the detector molecules of the different sets and the target molecules, wherein the particular labelled detector molecules of at least two sets bond in such a manner to a certain region of the target molecules that the different labels of the detector molecules overlap, and

(b) qualitatively and quantitatively evaluating the bondings obtained in this manner via the different labels of the detector molecules.

The term "biopolymers as target molecules" means DNA (preferably chromosomal DNA), RNA, or polypeptides. The target molecules may be appropriately arranged or immobilized prior to carrying out the method of invention, in particular prior to step (a), *e.g.*, by gel electrophoretic separation in a suitable matrix or fixing or arranging, *e.g.*, metaphase chromosomes or interphase nuclei on a suitable carrier.

The term "labelled detector molecules" means nucleic acids or antibodies having at least one label. The antibodies may be present polyclonally or monoclonally. The terms "nucleic acid" and "nucleic-acid sequence" and "nucleic-acid probes" mean native, semisynthetic, or modified nucleic-acid molecules of deoxyribonucleotides and/or ribonucleotides and/or modified nucleotides such as amino nucleotides or (α-S)-triphosphate nucleotides. In a preferred

embodiment of the current invention the nucleic acids stem from chromosomal DNA from, *e.g.*, mammals such as *homo sapiens sapiens*. The chromosomal DNA as detector molecules is present in vectors, *e.g.*, cosmids or YACs, or stems from chromosomal or chromosome region-specific DNA libraries which can be obtained, *e.g.*, via microdissection methods or laser-activated flow-cytometric sorting of specific chromosomes and, if required, subsequent amplification by, *e.g.*, DOP-PCR.

The term "labels" means suitable directly or indirectly detectable atoms or molecules which are introduced into the detector molecules or connected to them. Suitable labels are, *e.g.*, those comprising fluorescent dyes coupled to nucleotides and/or those comprising, *e.g.*, biotin and/or digoxigenin and/or nucleotides labelled with radioactive isotopes. In a preferred embodiment the labelling compound is a fluorescent dye having a difference, sufficient for the selection of small amounts of substance, in the fluorescence behavior of the emission spectra such as, *e.g.*, cumarins and rodamins, and/or in the fluorescence lifespan such as, *e.g.*, fluorescent isothiocyanates and europium-chelate-labelled and/or porphyrin-labelled avidines.

The term "bonding reaction" means a hybridization, preferably an in situ hybridization, or an antigen/antibody reaction dependent on the selection of the detector molecules and/or of the target molecules. The term "in situ hybridization" means the apposition of a synthetically produced DNA and/or RNA molecule provided with biological, physical or chemical labels for detection as detector molecule to native DNA and/or RNA sequences occurring in nature, wherein the apposition is achieved by denaturing and renaturing the appropriate nucleic acids. Of course, these DNA and/or RNA probes contain at least one sequence section capable of hybridizing with a DNA and/or RNA sequence of the target molecule, such as a chromosome.

This sequence section comprises a specific, individually present sequence region of the detector molecule which region is preferably 100 to 1,000 base pairs long and which apposes itself to a complementary region of the target molecule through the formation of hydrogen bridges at a suitable temperature, preferably at 50 °C or less, and at a suitable saline concentration comprising preferably 50-300 mmol/l monovalent ions and 0-10 mmol/l bivalent ions. The bonding reaction of the particular sets of labelled detector molecules may be carried out simultaneously or successively.

The expression "set of detector molecules" means detector molecules which are specific for a certain region of the target molecules. This set of detector molecules may be, *e.g.*, chromosomal DNA present in vectors or may be a chromosome-specific DNA library. The labels of the detector molecules in the set may be the same or different, *e.g.*, three different labels.

The expression "at least two or more different sets of labelled detector molecules" means the presence of at least one pair of different sets, wherein the sets of this pair bond in a certain area or region of the target molecules in such a manner that at least the different labels of the particular detector molecules, preferably the bonding sites of the particular detector molecules of these different sets, overlap. This property, according to the invention, of a pair of different sets means that the particular detector molecules in the different sets of a pair which are produced or obtained in an overlapping manner from this certain region of the target molecules can be used as a standard or for comparative examination with appropriately processed specimens from patients. In an embodiment according to the invention the detector molecules of a set are preferably designed in such a manner that after the hybridization the detector

molecules are bound in a continuously changed concentration, preferably in the manner of a Gauss distribution, in the longitudinal direction to the target molecules, *e.g.*, chromosomes.

The qualitative and quantitative evaluation of the bondings obtained in step (a) via the different labels of the detector molecules, which evaluation is characterized in step (b) of the method according to the invention, may be accomplished by employing a scanning device or a device for directed scanning, *e.g.*, along or in the longitudinal direction of the chromosome to be investigated. Such a scanning device is, *e.g.*, a fluorescence microscope. Image-generating signals can be taken by the scanning device via an image processing unit, *e.g.*, a CCD camera, via the physical and/or chemical and/or biological labels of the detector molecules which have been apposed to the desired target molecules. This image processing unit processes the individual signals of the different labels in a suitable manner supported by a computer. The intensities and/or the intensity relationships of the different labels in the regions of overlapping and non-overlapping labels of the particular detector molecules can be recorded and evaluated qualitatively and quantitatively, preferably in the longitudinal direction of the target molecules, particularly of fixed metaphase chromosomes, with this image processing unit coupled to the scanning device.

Further subject matter of the current invention comprises a diagnostic kit for the identification of changes in biopolymers, as defined herein, as target molecules, the kit containing at least two different sets of labelled detector molecules in accordance with definitions set forth above.

In particular, the kit according to the invention can be used for the identification or exclusion of chromosomal aberrations in human genetics such as balanced chromosome

rearrangements which are, as is known, of great significance for (a) the decision whether or not to have children, in the case of carriers of such a change; (b) balanced and unbalanced chromosome changes as the cause of malformations and/or mental retardation; and (c) in the tumor diagnosis of solid tumors as well as of hematological neoplasias (AML, ALL, MDS, and others), on the one hand for the detection of known alterations relevant to prognosis and on the other hand for the determination of further, previously unknown alterations.

Further subject matter of the current invention relates to an automatic imaging correction by addition of a localized DNA probe.

A monochrome CCD camera in combination with specific fluorescence filters is used when recording chromosome region-specific specimens labelled with different fluorochromes like the specimens used for the methods of multicolor banding. The signals of the individual fluorochromes are recorded successively as individual images and subsequently combined to a color image. A shift of the position of the individual images relative to each other on account of optical influences of the filters (different wedge errors, parallel shift due to slight tilting in the path of the rays) can not be excluded thereby. An interactive or automatic correction, *e.g.*, by a correlation of the individual images, is not possible with the required precision because the at most partially overlapping probes do not have any common structures which can be used for a subsequent superpositioning. Every slight shift results in the evaluation of the intensity ratios in artifacts in the banding pattern.

An automatic correction is made possible by adding a localized DNA probe which is simultaneously labelled with all fluorochromes used in the method according to the invention. A structure which is identical in all individual images is therefore available for the automatic

correction of position. The correction of position may take place, *e.g.*, via a determination of the center of intensity of the probe in each individual image and by a subsequent relative shifting of the individual images in such a manner that the centers of the individual images come to be located at the same position.

Additionally, distortions of the images relative to each other can be determined and corrected by the use of two different multi-labelled probes.

The use of even more probes basically makes possible the correction of more complex transformations of position than translation and rotation such as, *e.g.*, changes of scale.

Furthermore, it can be advantageous in a further preferred embodiment of the current invention to add calibrating probes (DNA probes or fluorescent particles) of known intensity which can serve for standardizing the intensities of the fluorescent signals to be evaluated.

Furthermore, it can be advantageous in a further preferred embodiment of the current invention to add DNA probes whose exact localization within the genome is known and which can be used for establishing the relation between color bands and the ISCN bands.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photographic representation for the qualitative and quantitative evaluation of the localization of region-specific colorations in chromosome 5. In the upper part of this figure the distribution of the labelled detector molecules in the longitudinal direction of the chromosome as well as intensities of the different labels of the detector molecules are shown graphically.

Figure 2 is a tabular presentation of the label pattern of the region-specific chromosome section of chromosome 5 shown in figure 1. Cy5, TR (Texas red), Cy5.5, SO (spectrum

orange), SG (spectrum green) are the different fluorescent dyes which were used to label the individual region-specific DNA libraries. The association is characterized by a solid square: (■). The labellings resulting from the overlapping of the DNA libraries in the corresponding regions are rendered recognizable by an empty square (□).

Figure 3 shows the respective homologous normal chromosomes 5 from two different metaphase plates with multicolored banding. The presentation makes it clear that the banding pattern is identical on the homologous chromosomes and can even be reproduced from metaphase plate to metaphase plate.

Figure 4 shows a photographic representation of a multicolored FISH of a metaphase plate with complex chromosomal aberrations.

Figure 5 shows chromosomes 5 in a case of acute myeloid leukemia. The normal chromosome 5 is shown on the right side and the chromosome on the left side displays an interstitial deletion in the long arm.

EXAMPLE

The following example explains the invention.

A total of seven overlapping chromosome microdissection region-specific libraries were produced for the multicolor band pattern of chromosome 5 (Meltzer et al., Nature Genet. 1, 1992, 24-26). The *p* arm of chromosome 5 was subdivided for this into two regions, the *q* arm into four regions. Eight to ten fragments per chromosome region were isolated with a finely drawn-out glass needle from the microscope slide under microscopic view (Senger et al., Hum, Genet. 84, 1990, 507-511). The thus obtained DNA was amplified via a DOP-PCR (degenerate

oligonucleotide polymerase chain reaction, Telenius et al., Genomics 13, 1992, 718-725; Zhang et al., Blood 81, 1993, 3365-3371). In a subsequent reaction these chromosome region-specific DNA libraries were partially labelled directly with fluorochromes coupled to nucleotides (*e.g.*, Spectrum Orange-dUTP, Spectrum Green-dUTP, both Vysis and Texas Red-dUTP, Molecular Probe). In another part, DNA libraries were labelled with nucleotides coupled to haptenes (*e.g.*, biotin-dUTP and digoxigenin-dUTP, Boehringer, Mannheim). After the hybridization has taken place haptenes can be detected with suitable detection reagents (*e.g.*, avidine-Cy5, Amersham, and anti-digoxigenin IgG, Boehringer, Mannheim, which is coupled to Cy5.5, Mab labeling kit, Amersham).

The hybridization, washing steps, and detection are carried out according to standard protocols (Senger et al., Cytogenet. Cell Genet. 54, 1993, 49-53).

The analysis is carried out, *e.g.*, with a fluorescence microscope equipped with suitable filter sets. Separate images are taken for each color channel, which images can be subsequently processed further with a computer.

A characteristic feature of the partial "painting" probes obtained by microdissection is a continuously weakening fluorescent signal in the border regions. A simultaneous overlapping of the probes and, therefore, of the fluorescent signals of two adjacent partial "painting" probes brings about a constantly changing ratio of the fluorescence intensities along chromosome 5. If a chromosome stained in this manner is subdivided into several (20-25) small sections, a false color stain can be assigned to each of these sections via a suitable computer program on the basis of the relative fluorescence intensities of all fluorochromes used. This assignment gives rise to a colored band pattern along a chromosome, in this case chromosome 5. The same combination

of fluorescence relationships and false colors can be used for all further hybridizations with the same specimen set.

Since the hybridization behaves in a sufficiently constant manner the band pattern is also correspondingly reproducible (figure 3). A loss of the resolving power in the case of shorter chromosomes, as is known from previous customary banding methods (*e.g.*, GTG banding) is not observed in this case. A reproducible pattern of at least 25 bands is achieved for chromosome 5. This corresponds to a band level of approximately 550 bands per haploid chromosome set.

It is possible with the aid of this method to identify changes in chromosomes independently of their condensation state. This is particularly significant in tumor cytogenetics, too. Tumor chromosomes often display a low resolution of the band pattern, which makes the recognition of chromosomal changes significantly difficult. It is therefore to be assumed that previously unknown cytogenetic changes are present in tumors, which possibly represent an important prognosis factor and could therefore be of significance, *e.g.*, for a risk-adapted therapy. According to the invention at least 25 bands can be achieved even on tumor chromosomes after hybridization with the specimen set for chromosome 5 described in detail above (figure 5).